Functional Consequences of Toll-like Receptor 4 Polymorphisms

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Toll-like receptor 4 (TLR4) is an important pathogen recognition receptor that recognizes mainly lipopolysaccharide (LPS) of Gram-negative bacteria, but also structures from fungal and mycobacterial pathogens, as well as endogenous ligands. Two nonsynonymous polymorphisms of TLR4, Asp299Gly and Thr399Ile, have been suggested to alter the function of the receptor. Some, but not all, studies have proposed that these polymorphisms lead to reduced cytokine response and increased susceptibility to Gram-negative infections. In this review, we compare studies that assessed the effect of the Asp299Gly and Thr399Ile polymorphisms on susceptibility to Gram-negative infections and examine the phenotypic consequences of these polymorphisms. In addition, we review the geographical distribution of TLR4 polymorphisms and present a model for evolutionary pressures on the TLR4 genetic make-up.
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INTRODUCTION

The innate immune system recognizes a broad range of pathogens and initiates protective responses. Recognition of pathogenic microorganisms by the innate immune system relies on pattern recognition receptors (PRRs) that detect preserved structures of bacteria, viruses, protozoa, and fungi, so-called pathogen-associated molecular patterns (PAMPs) (1).

A major group of PPRs are the Toll-like receptors (TLRs). After PAMP recognition, TLRs activate cellular signaling pathways to induce immune-response genes, including inflammatory cytokines (2). Ten different human TLRs have been identified (3). TLR11, which binds and recognizes uropathogenic bacteria and profilin in mice, has been shown to be nonfunctional in humans owing to a premature stop codon (4,5). Each TLR recognizes specific PAMPs, including recognition of lipoproteins, lipoteichoic acid, and zymosan by TLR2; dsRNA by TLR3; lipopolysaccharide (LPS) by TLR4; flagellin by TLR5; ssRNA by TLR7/8; and CpG DNA by TLR9 (1,6). TLR4, considered one of the most important PRRs, recognizes LPS of Gram-negative bacteria; mannans from fungal pathogens, a soluble component of *Mycobacterium* tuberculosis; and endogenous ligands, such as fibronectin and several heat shock proteins.

ROLE AND SIGNALING MECHANISM OF TLR4

We owe the elucidation of the function and genetic structure of TLR4 to the discovery of Toll, a transmembrane protein, initially discovered for its role in the embryonic dorsal-ventral development of *Drosophila* (7). *Drosophila* with a loss-of-function mutation for Toll exhibited a high susceptibility to fungal infection, demonstrating the importance of Toll in the antifungal response in the insect (8,9). After this discovery, work by Rock *et al.* (10) and Medzhitov *et al.* (11) independently led to the discovery of several human homolog genes for Toll, the so-called Toll-like receptors (TLRs).

Structure analysis of TLR4 revealed that the receptor consists of 3 domains: an extracellular leucine-rich-repeat (LRR) domain, a transmembrane domain, and an intracellular Toll-interleukin-1 receptor (TIR) domain. The extracellular LRR part of the receptor is involved in the binding of LPS. The discovery that LPS is a ligand for TLR4 came from studies in mice. Mice challenged with high doses of LPS normally develop a shock-like state similar to Gram-negative septic shock. More than 30 years ago two mouse strains, C_3H/HeJ and C57Bl/10ScCr, were discovered that were resistant to LPS (12). The phenotype was thought to be caused by a mutation in a hypothetical *lps* gene, but the location and function of this gene remained elusive for a long period (13). The groundbreaking studies of Poltorak and colleagues (14,15) in 1998 identified TLR4 as the *lps* gene and demonstrated that TLR4 is the LPS sensor in both mice and humans.

Overall, recognition of LPS and initiation of signaling by TLR4 is a complex process, which involves several accessory proteins. LPS is first bound by circulating lipopolysaccharide-binding protein (LBP), which functions as an opsonin for CD14 (16). CD14 then acts as a

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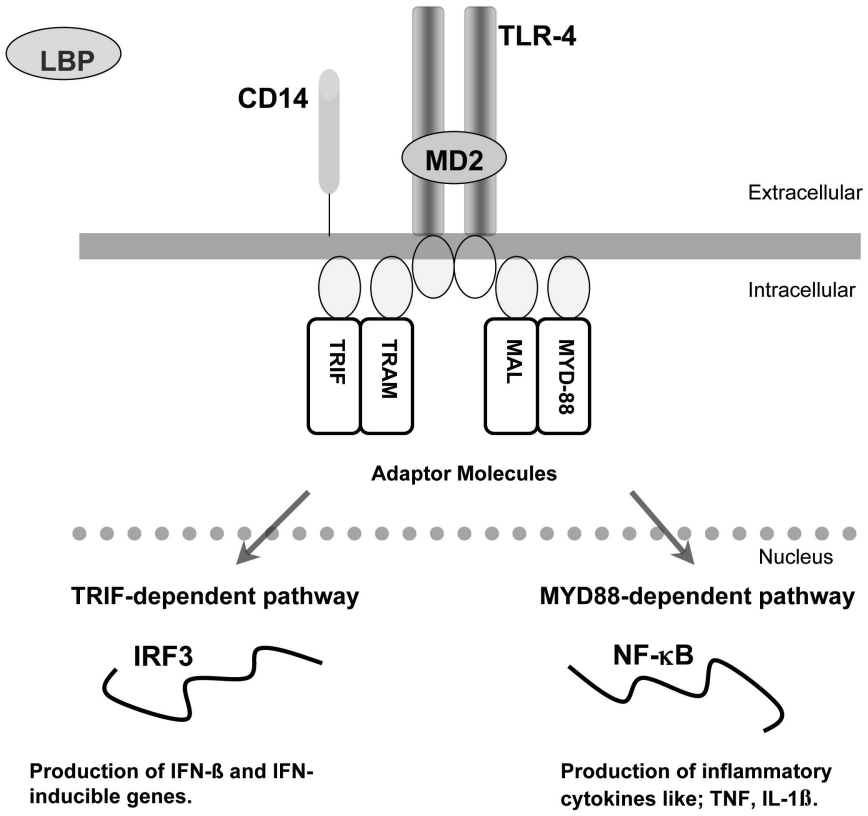

Figure 1. Schematic overview of the LPS TLR4 signaling pathway. Recognition of LPS is established by the interplay between lipopolysaccharide-binding proteins (LBPs) and CD14 that transfer the LPS to the MD-2/TLR4 complex. Dimerization of TLR4 then initiates downstream intracellular signaling transduction through Toll-interleukin-1 receptor (TIR) domains and several adaptor molecules. Eventually, the intracellular signaling results in the activation of specific pro-inflammatory cytokine profiles by the MYD88-dependent or TRIF-dependent pathway.

catalyst for the binding of LPS to MD-2 (17-19). After the LPS is transferred to MD-2, the LPS/MD-2 complex interacts with TLR4. Recent MD-2/TLR4 crystallography studies have elucidated the structures of this complex (20). Formation of the LPS/MD-2/TLR4 complex eventually results in the dimerization of the TLR4 TIR domain, initiating downstream signaling transduction (21).

Downstream signaling by the TLR4 receptor involves several intercellular TIR domain-containing adaptors mediating proinflammatory gene expression (22,23). Two pathways that initiate downstream TLR4 signaling are known, namely the MyD88- and TRIF-dependent pathways (see Figure 1). One pathway is mediated by myeloid differentiation factor 88 (MyD88) and the TIR domain-containing adaptor protein, also called MyD88 adapter-like protein (TIRAP/MAL). Initiation of MyD88-dependent pathway activation leads to the activation of nuclear factor κB (NF-κB) and the transcription of proinflammatory genes (24). A second pathway is mediated by the TIR-containing protein, also called TRIF-related adapter molecule (TIRP/TRAM), and TIR domain-containing adapter inducing interferon-β (TRIF). Initiation of the TRIF-dependent pathway leads to the activation of interferon regulatory factor 3 (IRF3), and the expression of interferon (IFN)-β and IFN-inducible genes (21).

Much research has been performed to understand the signaling pathways and innate immune response against Gram-negative infections mediated by TLR4, which is therefore one of the best-understood Toll-like receptors. Because of this, TLR4 also provides an ideal model to study the consequences of genetic variation and their relation to the function of the receptor, and their susceptibility to diseases. Here we review several genetic polymorphisms of the TLR4 receptor. Also, we try to understand if there are any TLR4 genotypes that result in phenotypic consequences.

GENETIC VARIATION IN HUMAN TLR4

Recognition of pathogens and downstream signaling through innate pathways involving the TIR domain show high conservation between organisms (19). This is in line with the finding that the innate immune system shows a high degree of homology between mammals, insects, and plants, and apparently has been highly preserved during evolution (25). Genetic variation between the TLR4s of different mammals is the highest in the extracellular LRR region (26). The stronger variation in this region, which is involved in the recognition of PAMPs, is probably the result of the evolutionary pressure by pathogens on the host.

Sequencing of the human TLR4 revealed that most of the variation in nonsynonymous polymorphisms is located in the third exon, coding for the LRR domain (27). Despite this greater variation in the TLR4 LRR domain, the frequency of most nonsynonymous polymorphisms is low in human populations (<1%). Exceptions are 2 nonsynonymous polymorphisms (SNPs) that have been described with population frequencies >5%. These are an A/G transition causing an aspartic acid/glycine substitution at amino acid location Asp299Gly (rs4986790), and a C/T transition causing a threonine/isoleucine switch at amino acid location Thr399Ile (rs4986791). Arbour *et al.* (28) were the first to report that individuals with either the Asp299Gly and/or Thr399Ile polymorphisms had a blunted response toward inhaled LPS.

The finding that TLR4 polymorphisms Asp299Gly and Thr399Ile had an effect

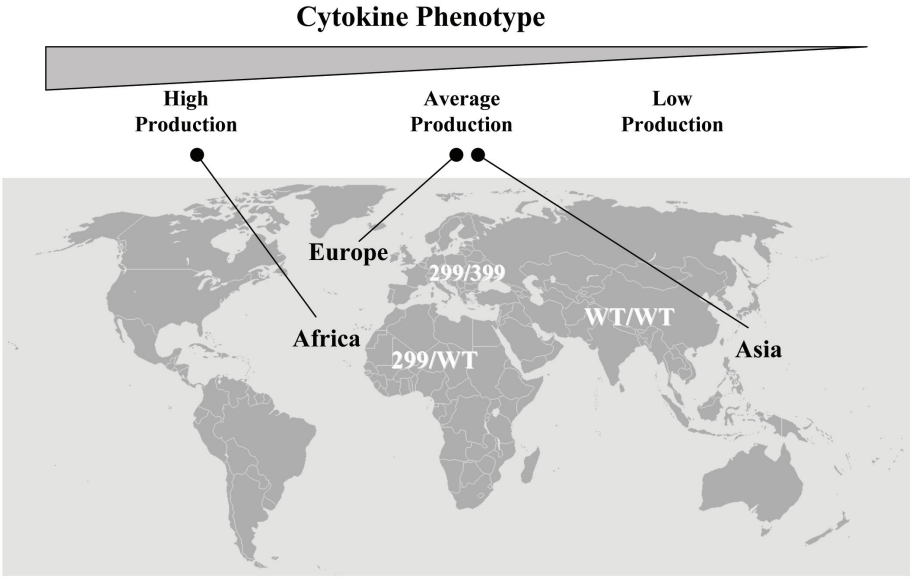

Figure 2. Distribution of TLR4 haplotypes and associated cytokine phenotypes across the 3 continents of the Old World. It shows that Africa, Asia, and Europe each have their own distinct haplotype, based on the alleles found, shown in white. The phenotypic end point is shown on the top of the figure, where the expected cytokine production following TLR4 stimulations is presented. When the geographic distribution of Asp299Gly and Thr399Ile polymorphisms is taken into account, it illustrates that research preformed in Europe has looked at the cosegregated Asp299Gly/Thr399Ile haplotype, whose cytokine phenotype does not differ from the wild-type TLR4 cytokine response. This also explains the large number of studies, performed in Europe, that did not find any association between TLR4 polymorphisms and susceptibility to disease. In contrast, the Asp299Gly/WT haplotype, that is found almost exclusively in Africa, has a stronger pro-inflammatory cytokine response compared with wild-type TLR4.

on the responsiveness to bacterial endotoxin initiated many genetic association studies. The results of these studies led to contradictory conclusions about the role of the Asp299Gly polymorphism and its effect on susceptibility to Gram-negative bacterial infections (29). This raises the question of the source of these discrepancies. One possible explanation may be that these studies looked at the Asp299Gly and Thr399Ile polymorphisms separately. Most studies either deal with the Asp299Gly or the Thr399Ile polymorphism, but neglect the fact that these polymorphisms also exist in a cosegregated (Asp299Gly/Thr399Ile) way (27). This cosegregated state of TLR4 implies that 4 haplotypes, namely wt/wt, Asp299Gly/wt, Thr399Ile/wt, and Asp299Gly/Thr399Ile, are represented in the population. Our results on cytokine profiles in LPS-stimulated whole blood cultures demonstrated that only the Asp299Gly haplotype differs in phenotype, showing an increased, rather than a blunted, pro-inflammatory tumor necrosis factor (TNF)-α response (30). Interestingly, the LPS-induced cytokine response in the Asp299Gly/Thr399Ile haplotype does not differ from the wild-type TLR4 cytokine response (30). Because the single Thr399Ile haplotype is rare, its phenotype is still unknown. Therefore, the TLR4 haplotype that alters the cytokine response to LPS (and thus could affect the susceptibility to Gram-negative infection) contains the single TLR4 Asp299Gly mutation (Figure 2).

Population studies also revealed a specific geographical distribution of the TLR4 haplotypes (27). All haplotypes trace their origin to before the out-of-Africa migration approximately 55,000 to 60,000 years ago. Nowadays, human populations from Africa have a 10- to 20-fold higher frequency of the Asp299Gly haplotype than those from the other continents. In contrast, white populations show an almost complete absence of the Asp299Gly haplotype, but have a higher frequency of the Asp299Gly/Thr399Ile haplotype. Asian populations lack all three of the Asp299Gly, Thr399Ile, and Asp299Gly/Thr399Ile haplotypes (Figure 2). This pattern of TLR4 haplotypes has been the result of differences in environmental pathogenic pressure on the human population during the migration into Eurasia, Europe, Asia, and the New World (30). As a consequence of this specific geographical distribution, most published studies performed in populations with a European genetic background have analyzed the Asp299Gly/399 haplotype, with no distinct phenotype, instead of the Asp299Gly haplotype. To a certain extent, the genetic background of the studied populations could explain the lack of agreement between published TLR4 polymorphism studies. We have therefore reanalyzed published data on the Asp299Gly/Thr399Ile and Asp299Gly haplotypes separately, and tested whether the presented Asp299Gly and Asp299Gly/Thr399Ile haplotypes correlate with the presented phenotypes.

FUNCTIONAL CONSEQUENCES OF THE COSEGREGATED ASP299GLY/THR399ILE POLYMORPHISMS

Terms used for a PubMed search were TLR4 polymorphisms, Asp299Gly, and Toll-like receptor 4 polymorphisms, which resulted in 164 articles after exclusion of all reviews. These articles studied the relationship between TLR4 polymorphisms and susceptibility to sepsis, atherosclerotic disease, asthma, *Candida* infections, chronic periodontitis, respiratory syncytial virus (RSV), transplantation, and Crohn's disease (31-51). The pathogenic ligand in most of these studies is based on the Gram-negative bacterial LPS, with the exception of the *Candida* and RSV studies.

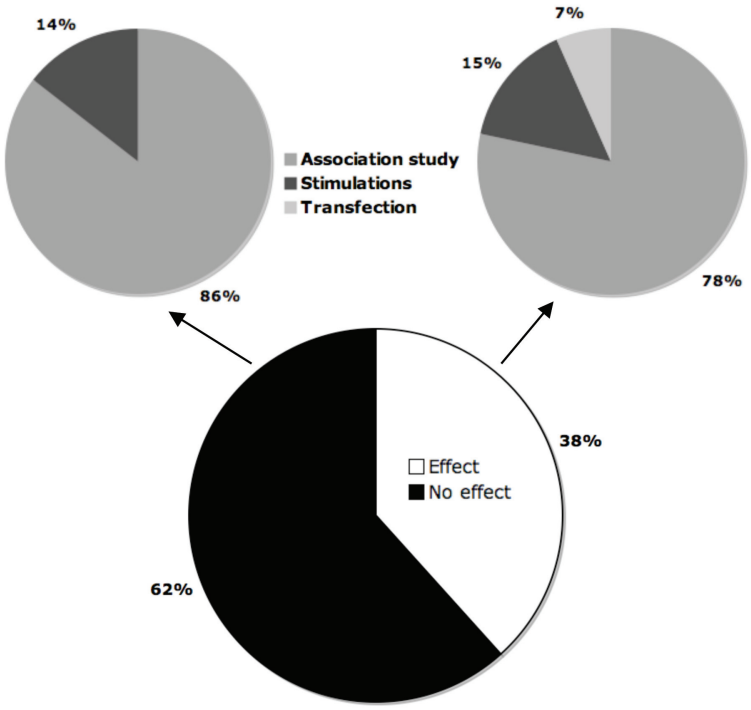

Figure 3. Circle diagrams with the frequency of the study results and outcomes (below) of TLR4 polymorphism from the PubMed search. This search revealed that more than half of the studies do not find any association between susceptibility to infection and TLR4 polymorphisms (n = 157). In the top diagrams, the frequencies of the methodologies used are presented in the studies that showed a positive effect or no association of the investigated TLR4 polymorphisms. This figure shows that the majority of studies are based on genetic association studies, and a minority measured cytokine production following LPS stimulation of either whole blood or PBMCs. Overall, the contrast between study outcomes measuring the functional effects of TLR4 polymorphisms and of those looking at the role these have in susceptibility is revealed.

The strongest association to date between TLR4 polymorphisms and disease susceptibility has been reported in RVS infections (52), but the functional relationship between TLR4 polymorphisms and RSV virus remains to be elucidated. Because specific parts of the LRR involved in the recognition of various ligands can differ, only the LPS studies were included. This resulted in 157 research articles about the effect of the TLR4 Asp299Gly and Asp299Gly/Thr399Ile SNPs. Of these articles, 62% reported no association between the susceptibility to disease and TLR4 polymorphisms (see Figure 3). The majority of articles included European populations (90%), and the rest screened Asian (6%) or African (4%) populations. The consequence of the studies involving European populations is in agreement with the suspicion that these studies did not look at the Asp299Gly haplotype, as they state, but reported de facto on the effect of the Asp299Gly/Thr399Ile haplotype. Besides the population origin, study methodology can also influence results. For the TLR4 studies, three types of methodology can be distinguished: genetic association studies, functional stimulation experiments, and cloning and transfections of the mutated TLR4. Figure 3 shows that the majority of published articles are studies of genetic associations. Most of the association studies on TLR4 polymorphisms are small and underpowered, whereas larger studies tend to be negative and find no correlation between the TLR4 Asp299Gly/Thr399Ile haplotype and disease susceptibility (53).

Although genetic association studies find no correlation between haplotypes and disease strictly, they do not give insight into the direct functional consequences of TLR4 mutation. Several studies have investigated the functional consequence of TLR4 SNPs, and the results are summarized in Table 1. The studies of Arbour *et al.* (28) and Schwartz (54-56) show that transfected cells with any of the TLR4 haplotypes have a decreased NF-κB activity compared with normal TLR4. This suggest that the Asp299Gly/Thr399Ile haplotype should have an effect on phenotype, leading to reduced cytokine production by the innate immune response. The question then is: why are the consequences of such a functional effect not observed in genetic association studies?

One reason is that the use of transfection to investigate the phenotypic effect of certain mutations meets with several complications. Transfections represent a stripped-down model performed in altered cell lines. As a result, transfection measurements do not give the ideal end point of phenotypic difference representing the whole system. Transfections only represent the homozygous state. Most population studies of genetic association and function include TLR4 haplotypes in heterozygous state, whereas the homozygous state of these polymorphisms is rare. Disruptions of the receptor transport can influence results by overfeeding the system (28). Therefore, determination of the association between the Asp299Gly/Thr399Ile haplotype and the phenotype, and its impact on the susceptibility to Gram-negative infection (based on transfection experiments), is difficult and prone to artifacts.

Besides transfection experiments, most functional studies are based on *in vitro* stimulation of either whole blood or peripheral blood mononuclear cells

Table 1. TLR4 polymorphisms and their impact.

	Association	Stimulation	Transfection	Effect of polymorphism
Calvano *et al.* (57)	Systemic endotoxemia	Whole blood *in vivo*		No
Kumpf *et al.* (65)	Pre- and postoperative cytokines	Whole blood		No
Schippers *et al.* (59,60)	*In vivo* and *ex vivo* response	Whole blood		No
Newport *et al.* (62)	Pulmonary tuberculosis	Whole blood		No
Heesen *et al.* (66)	Cytokine synthesis	Whole blood		No
Von Aulock *et al.* (67)	LPS-induced cytokine release	Whole blood		No
Chang *et al.* (68)	TLR4 in peripheral neutrophils and monotypes	Whole blood		No
Marsik *et al.* (58)	Endotoxemia	Whole blood *in vivo*		Yes
Tiberio *et al.* (69)	Innate response to candidate adjuvants RC529 and monophosphoryl lipid A	Whole blood		No
Kroner *et al.* (70)	Multiple sclerosis	PBMCs		No
Van der Graaf *et al.* (71)	Stimulations with different TLR4 ligands	PBMCs		No
Peeters *et al.* (51)	Impact of polymorphisms on monocytes in patients with Crohn's disease	PBMCs		No
Fagerås *et al.* (72)	Asthma	PBMCs		Yes
Erridge *et al.* (73)	LPS-induced monocytes signaling (monocytes)	PBMCs		Yes
Schmitt *et al.* (74)	Response to LPS	PBMCs		Yes
Norata *et al.* (75)	Intima-media thickness and LPS monocyte-derived macrophage response	PBMCs		No
Montes *et al.* (76)	Hematogeneous osteomyelitis	Neutrophils		Yes
Kinane *et al.* (77)	Porphyromonas gingivalis	Epithelial		Yes
Schwartz (54-56)	Inhaled LPS	Airway epithelia	THP-1	Yes
Arbour *et al.* (28)	Inhaled LPS	Airway epithelia	THP-1	Yes
Rallabhandi *et al.* (64)	Insights in stoichiometry, structure, and signaling		HEK-239T	Yes

(PBMCs) stimulated with LPS. After stimulation, cytokine release by the cells is measured. The whole blood cultures and experiments using isolated PBMCs are in agreement with the association studies, as they find little difference between Asp299Gly/Thr399Ile and the wild-type haplotype (Table 1). Besides the *in vitro* experiments, three *in vivo* studies investigated the effects of LPS in human volunteers. Two of these *in vivo* experiments injected 2 ng/kg LPS (57,58). Neither of them found a difference in the TNF-α serum concentration between individuals with different TLR4 haplotypes. However, one study found decreased IL-6 levels after 6 h in volunteers with TLR4 polymorphisms, whereas the other did not find any differences (57,58). The former study also reported strongly decreased IL-1β production after 24-h stimulation, suggesting an effect on the late TRIF-dependent pathway (58). A third paper measuring *ex vivo* and *in vivo* cytokine production of the different TLR4 haplotypes also did not find differences between Asp299Gly/Thr399Ile and the wild-type haplotypes (57,59,60). Thus, most studies of stimulated whole blood and PBMCs do not find a distinct phenotype of the Asp299Gly/Thr399Ile haplotypes.

FUNCTIONAL CONSEQUENCES OF THE ASP299GLY POLYMORPHISM

As mentioned above, studies that include or define the Asp299Gly haplotype are scarce. The best studies of the Asp299Gly haplotype are those performed in Africa. Unfortunately, no association studies performed in Africa have focused specifically on the response to TLR4 ligands. The study by Mockenhaupt *et al.* (61) focused on malaria, whereas that of Newport *et al.* (62) looked at susceptibility to *Mycobacterium tuberculosis*. Neither study included stimulated cytokine production, and therefore it is impossible to say anything about the LPS responses in the Asp299Gly haplotypes. Although these studies indicate a role for the Asp299Gly haplotype in susceptibility to malaria and tuberculosis, they do not reveal the role of the haplotype in the recognition of Gram-negative bacteria and LPS.

In contrast, Lorenz *et al.* (63) looked at sepsis patients versus healthy controls in France and specifically defined the Asp299Gly haplotype. The Asp299Gly

haplotype was found only in the group of patients with septic shock, whereas the Asp299Gly/Thr399Ile haplotype was found equally in both patients and controls. This suggests that the Asp299Gly haplotype has an effect on phenotype, although cytokine measurements are lacking. Until now, only one study reported cytokine production by individuals with the Asp299Gly haplotype—it found a stronger, rather than a blunted, TNF-α cytokine response (30). Although more research has to be performed to settle the functional consequences of the Asp299Gly haplotype, the evidence so far indicates that the TLR4 wild-type haplotype and the Asp299Gly haplotype have different phenotypes.

EFFECTS OF THE ASP299GLY ON THE 3-DIMENSIONAL STRUCTURE OF TLR4

Crystallography of the TLR4-LPS/MD-2 complex reveals that there are two highly preserved regions of TLR4 involved in the binding of the LPS/MD-2 complex (20). These preserved domains are located at the N-terminal and central domain of the receptor (20). The crystallography shows that the Asp299Gly is not directly involved in the binding of MD-2, but the mutation is located close to the TLR4-MD-2 binding area. Although there is no direct alteration of the binding location of the LPS/MD-2 complex, it has been suggested using an ectodomain model of TLR4 that Asp299Gly polymorphism increases the rotational freedom of the peptide bond (64). Interestingly, the same model also shows that the wild-type TLR4 has a negatively charged area at position 299, and this is lost in the Asp299Gly (64). The modified response of cells of Asp299Gly polymorphism individuals could therefore be the result of the increased rotation and change of charge that may modulate the interaction of LPS with the TLR4 receptor.

CONCLUSION

It is tempting to conclude that TLR4 polymorphisms have an effect on the susceptibility to infections, especially because of their location in the ligand recognition area of the receptor.

Despite the tempting theoretical considerations and a number of reports based on *in vitro* experiments, most if not all of the studies using primary cells isolated from individuals bearing the mutation reveal that the Asp299Gly/Thr399Ile haplotype has little or no effect on responsiveness to LPS, and the susceptibility to infections of the bearer is unchanged. Therefore, we tend to conclude that the Asp299Gly/Thr399Ile haplotype has no distinct phenotype. In contrast, the Asp299Gly haplotype exhibits a stronger pro-inflammatory TNF-α cytokine response after stimulation with LPS. In addition, this phenotype seems to predispose to septic shock. The effect of the rare Thr399Ile haplotype on function and susceptibility remains unclear due to its scarcity in the population.

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